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Organic field-effect transistor-based biosensors functionalized with protein receptors

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An organic field-effect transistor with integrated proteins (Bio-FET) for sensing of sulfate ions is presented. A sulfate receptor was engineered to contain a thiol group for surface-anchoring without affecting its binding activity. The modified receptor was covalently coupled to a maleimide-functionalized polystyrene layer, and integrated as gate dielectric in a dual-gate transducer. The binding of sulfate ions in dry conditions was detected by a shift in the threshold voltage. Combined with surface density measurements by atomic force microscopy, an effective charge of $-1.7q$ per protein was found, as expected from the Bio-FET operation model. © 2010 American Institute of Physics. [doi:10.1063/1.3518681]

I. INTRODUCTION

A standard biosensor is composed of three parts:¹ the *biological detection component*, which can be a biological molecule or even a cell, the *transducer* which transforms the signal detected by the biological component into an electrical or luminescence change, and the *processing unit* which amplifies and/or filters the signal. A crucial property for any sensor is selectivity.² Usually the target analyte is present as a minor component in a mixture with many other species. A sensor with poor selectivity will not only detect the target analyte but also other compounds, greatly reducing the efficacy. In nature most biochemical processes use biomolecules such as nucleic acids or proteins that are selective. By incorporating these biomolecules in a transducer as a thin film transistor a selective biosensor (Bio-FET) can be achieved. Transistors based on organic materials are especially suited for this purpose, since these materials can be tailored and functionalized to bind specific biomolecules. Here we present a prototype Bio-FET sensor with integrated proteins as schematically depicted in Fig. 1(a). The bottom part is the transducer, an organic field-effect transistor consisting of a source and drain electrode, an organic semiconductor, a gate dielectric, and gate electrode. The semiconductor is protected by a thin insulating top layer, onto which a protein is attached. The purpose of this layer is twofold. First, it acts as a protection layer for the organic semiconductor. Second, the insulator is tailored with anchor groups to attach the receptor protein. In order to arrive at a selective sensor, we focused on a sulfate binding protein (SBP) that is associated with ATP-binding cassette transporters.^{3,4} These proteins confer high-affinity and selectivity on the transport process due to their dissociation constants (K_d) in the (sub)micromolar range and

have also been used in biosensor devices.⁵ Moreover, their structure has been extensively studied due to their well defined behavior during overexpression, purification, and crystallization trials.⁶

The operation principle of the Bio-FET is depicted in Fig. 1(b). As a first order approximation, the Bio-FET can be regarded as a dual-gate field-effect transistor⁷ where the top gate has been replaced by the bioreceptors. As shown in Fig. 1(b), upon application of a negative bias on the bottom gate the transistor accumulates positive charge at the bottom insulator-semiconductor interface. The green spheres on top of the device represent the protein receptors, which capture sulfate ions, hence trapping negative charges at the interface. The top insulating layer of the Bio-FET acts as a capacitor and positive counter charges are then accumulated in the semiconductor. In order to deplete the channel from these positive counter charges, a higher positive bias at the bottom gate is required to switch on the transistor. This effect can be measured as a shift in the threshold voltage as: $\Delta V_{TH} = Q_{REC}/C_B$, where Q_{REC} is the charge trapped at the interface and C_B is the bottom insulator capacitance.⁸

The challenge for the realization of the Bio-FET is the attachment of the analyte receptor to the surface of the top insulator. For this purpose we functionalized the insulator with maleimide side-chains, that can chemically bind to thiol groups. Since the wild type SBP does not contain any thiol group, it is functionalized with a thiol-containing cysteine group. In this way a covalent link between the thiol of the cysteine and the maleimide side-chains of the insulator can be established. Since many proteins contain cysteines in their amino-acid sequence, it might seem superfluous to engineer an additional cysteine into their structure. However, several (bacterial) receptor proteins, like SBP, do not contain cysteines in their amino-acid sequence.^{9,10} We note that even

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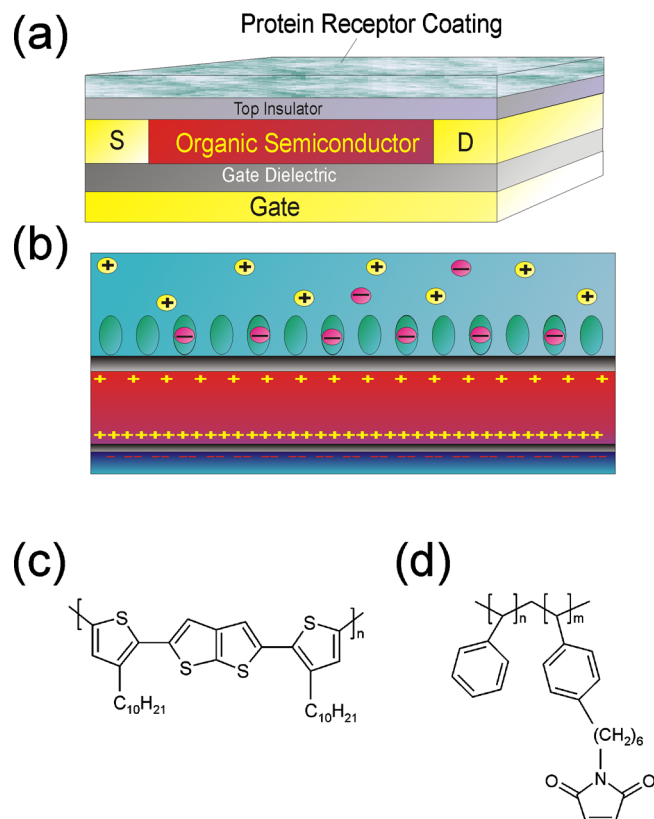


FIG. 1. (Color online) (a) Schematic view of the Bio-FET device. (b) Operation principle, including the binding of sulfate ions to the receptor and charge compensation in the semiconductor. (c) Chemical structure of the semiconductor, PDTT. (d) Chemical structure of the maleimide functionalized polystyrene, PSMI.

proteins that do contain cysteines will probably need to be engineered with an extra cysteine as well, since the cysteines in their original structure might be positioned in the inside of the protein structure or form disulfide cysteine-cysteine bridges as part of the structure. This would render the thiol group of the cysteine useless as a possible anchor for chemical coupling. Furthermore, the position of the cysteine should not be too close to the reactive center of the protein since the binding of the thiol group might interfere with the biochemical activity of the protein.¹¹ For these reasons, modification of the protein is often desirable or even required to anchor the protein to a surface.

Guided by the three-dimensional (3D)-structure of the protein¹⁰ (Fig. 2), we engineered a surface-exposed cysteine at position 289 of the protein, replacing a glycine.¹² The modification, G289C, was chosen in such manner not to disrupt the overall structure of the protein and relatively far away from the sulfate binding site. The modification did not affect the functionality of the protein as the modified SBP(G289C) binds sulfate with a K_d of 0.2 μM , similar to the K_d of the wild type protein. The K_d values of the proteins were determined according to a literature procedure.¹² The chemical structure of the modified insulator, polystyrene maleimide (PSMI), is shown in Fig. 1(d). In this paper, we demonstrate that the modified SBP binds to the top surface of the PSMI-insulating layer and that sulfate ions can be detected under dry conditions.

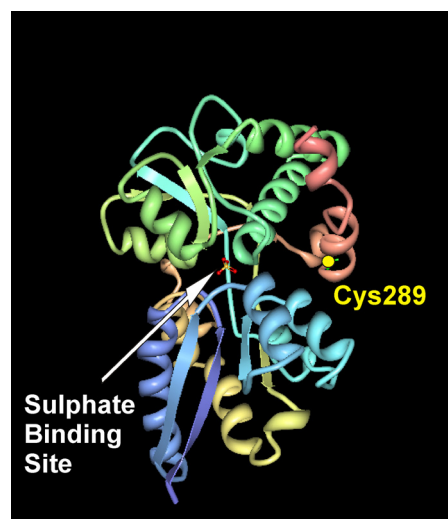


FIG. 2. (Color online) 3D structure of SBP with Cys-289 and analyte-binding site highlighted. (RCSB Protein Databank; Pflugrath, and Quirocho, 1988).

II. MATERIALS AND METHODS

A. Synthesis of the polymers

All reagents and solvents were purchased from Acros Organics or Sigma Aldrich and used without further purification, unless stated differently. We used poly(4,4'-didecylbithiophene-co-2,5-thieno[2,3-b]thiophene) (PDTT, structure shown in Fig. 1(c)) as a semiconductor since it is stable at ambient conditions.¹³ The monomers were synthesized according to published procedure^{13–15} and then copolymerized. The polymerization was performed under N_2 -atmosphere; the yield of polymerized PDTT was 90%. For the synthesis of PSMI, Dow Styron 683 polystyrene was used as a starting material and the functionalization was done as described previously.¹⁶ The amount of side-tails functionalized with maleimide was estimated between 20% (gel permeation chromatography) and 29% ($^1\text{H-NMR}$).

B. Expression and purification of SBP

The wild type and the G287C mutant of the SBP protein were expressed and purified as described previously.¹² To facilitate the purification of the proteins, a sequence encoding *tobacco etch virus* protease (TEV) cleavage site, followed by a decahistidine tag, was added to the 3' end of the gene. The proteins were isolated from *Escherichia coli* cell lysates and purified by metal-affinity chromatography (using Ni^{2+} -Sepharose resin), followed by TEV protease cleavage to remove the decahistidine tag.¹² Dowex X1/2, a strong basic anion resin, was used to remove contaminating sulfate from buffer (2 mM tris-HCl, $\text{pH}=7.5$) and protein solutions.

C. Fabrication and testing of the Bio-FET

Field-effect transistors were made on standard n^{++} -Si substrates with a 200 nm thermally grown oxide layer. Gold source and drain electrodes of 100 nm on a 5 nm Ti adhesion layer were defined by photolithography. The oxide layer was passivated with hexamethyldisilazane to prevent interface charge-trapping.¹⁷ PDTT was spincoated under

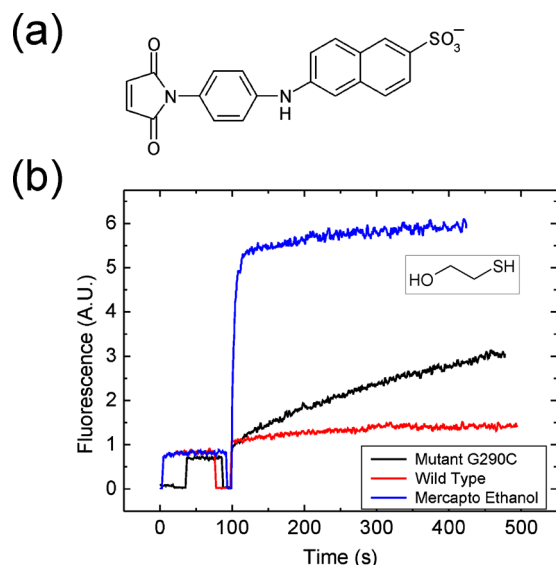


FIG. 3. (Color online) (a) Chemical structure of MAL-ANS. (b) Fluorescence intensity vs time upon incubation of MAL-ANS with 2-mercaptoethanol, wild type SBP and SBP(G289C). The inset shows the structure of 2-mercaptoethanol. The excitation wavelength was 328 nm.

N₂-atmosphere from 1,2-dichlorobenzene (1.5% w/v) and annealed at 125 °C for 30 min. PSMI was deposited by spin-coating from ethyl methylketone (3% w/v). For the protein coating, a 10 μ M SBP solution in 2 mM tris-HCl, pH 7.5 was incubated with the PSMI surface for 15 min and afterwards gently rinsed with demineralized water and spin-dried. Finally, to test the functionality of the protein-coated PSMI surface the Bio-FET transducer was immersed in an aqueous solution of 1 mM Na₂SO₄. The surface was not rinsed with demineralized water afterwards but only spin-dried.

III. RESULTS AND DISCUSSION

A. Fluorescence binding-assay

To confirm the accessibility of the thiol group in the cysteine group at position 289 of the protein for maleimide the fluorophore 2-(4'-maleimidyl-anilino)naphthalene-6-sulfonate [MAL-ANS; Fig. 3(a)], was used to probe the reactivity of Cys-289. Mal-ANS becomes highly fluorescent when its maleimide group reacts with a thiol group. The purified protein, wild type and SBP(G289C), were diluted at a concentration of 0.2 mM in 2 mM tris-HCl buffer, pH 8.0 at 20 °C, for a total volume of 1.990 ml. At time zero 10 μ l 10 mM MAL-ANS solution was added under continuous stirring. Figure 3(b) shows the fluorescence intensity versus time, reflecting the chemical ligation of the fluorophore to the protein. The extent of labeling of SBP(G289C) was estimated from the protein concentration and calibration of the fluorescence upon ligation of MAL-ANS to 2-mercaptoethanol, a small thiol-containing molecule. The labeling of SBP(G289C) was complete after 15 min and the extent of labeling was close to 100%. Control experiments with wild type SBP showed that the maleimide reacts specifically to Cys-289, as seen in Fig. 3(b).

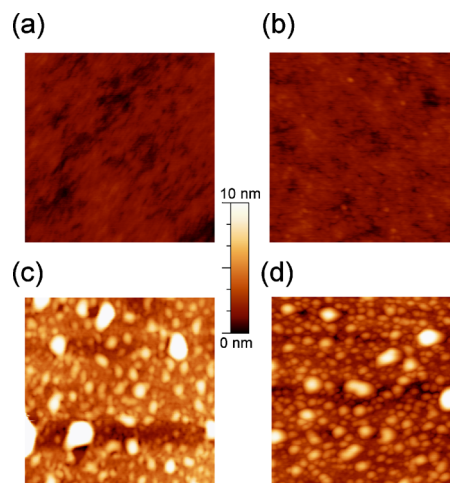


FIG. 4. (Color online) TM-AFM measurements of the PSMI surface; X/Y-range=500 \times 500 nm², Z-range=10 nm. (a) Pristine surface. (b) Surface after exposure to buffer, 2 mM tris-HCl, pH 7.5. (c) Surface after exposure to the SBP solution for 5 min. (d) Surface after exposure to the SBP solution for 10 min.

B. Atomic force microscopy measurements

The binding of SBP(G289C) to the maleimide-functionalized top surface of PSMI was investigated by tapping mode atomic force microscopy (TM-AFM). The height profiles are shown in Fig. 4. Images in Figs. 4(a) and 4(b) present the surface of PSMI before and after exposure to the buffer used to dilute the protein, for 15 min, respectively. The images are practically identical showing that the buffer leaves the PSMI surface unmodified. Figures 4(c) and 4(d) show PSMI exposed to the protein solution (10 μ M) in buffer for 5 and 10 min. The surface now shows globular corpuscles. The objects have diameters that range from 5 to 20 nm with a height of 3 to 6 nm. These dimensions agree reasonably well with those of a single SBP molecule, within the resolution limit of the AFM tip. The reported dimensions of this protein are 3.5 \times 3.5 \times 6.5 nm³.^{10,18} Figures 4(c) and 4(d), therefore, indicate that the proteins probably retain their shape and structure on the surface even after the solution was removed from the surface by spin-drying. We extracted an areal density, N_{REC} , of about 7 \times 10¹⁵ proteins/m². We note that some objects are too big to be single polypeptides, which could indicate formation of aggregates.

C. Stability of the protein to drying

A critical issue is the stability of a protein upon a surface, especially under dry conditions. To determine the stability of the protein we measured the dissociation constant¹⁹ for the pristine SBP and for SBP subjected to drying on a polymer and afterwards rehydrated with buffer. SBP can bind chromate ions, albeit with lesser affinity than sulfate ions and when this binding occurs, a quenching of the fluorescence of the protein can be observed. The dissociation constant for chromate ions (K_d [CrO₄²⁻]) was determined by measuring the decrease in fluorescence during the titration with chromate ions. Purified SBP, wild type or SBP(G289C), was diluted to a concentration of 0.5 μ M into 2 mM tris-HCl buffer, pH 8.0 at 20 °C for a total volume of 1 ml. Increasing

TABLE I. Dissociation constants of the wild type (WT) and the SBP(G289C) mutant, both for the pristine protein and the dried and subsequently rehydrated protein.

Sample	$K_d[\text{CrO}_4^{2-}]$ (μM)
WT pristine	2.78
WT dried	3.74
G289C pristine	2.70
G289C dried	4.02

amounts of chromate, 1–2 μl of a 1 mM chromate solution in buffer, were added under continuous stirring and the decrease in fluorescence was recorded on a Fluorolog 3 fluorospectrometer. The excitation and emission wavelengths used were 285 nm and 325 nm, respectively. The dissociation constants for the wild type SBP and SBP(G289C) are shown in Table I. We see that there is an increase in the dissociation constant of chromate for the dried protein with respect to the pristine protein, indicating that the affinity of the protein toward chromate ions is diminished by the drying of SBP. However, the data show that the proteins are still active even after drying and rehydration. This indicates that the wild type SBP and SBP(G289C), are both stable even under dry conditions. The drying process does not affect the affinity of the protein toward the analyte; the protein remains active even after being dried.

D. Electrical measurements of the Bio-FET

The transfer characteristics of the Bio-FET prototype are presented in Fig. 5. The black curve presents the pristine PDTT film in vacuum and dark. The mobility is $7.2 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at a gate bias of -20 V . The red curve shows the current transport of the Bio-FET, that is, with SBP(G289C) bound to the PDTT film. The exposure to buffer caused a small shift in the transfer characteristics, which could be due to trapped ions in the protein layer. The blue curve shows the transfer characteristics of the device with protein coating after exposure to sulfate ions from a 1 mM Na_2SO_4 solution. A significant shift in both pinch-off

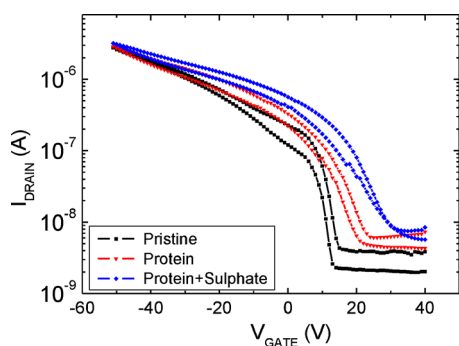


FIG. 5. (Color online) Transfer characteristics of Bio-FET transducers with $L/W=20/10\,000$ in the saturated regime, with PDTT as semiconductor coated with a 50 nm PSMI Layer.

and threshold voltage was observed. The shift in the threshold voltage was -11 V . The sign is consistent with the presence of a negative charge at the surface of the PSMI layer. Because the Bio-FET was incubated under 1 mM sulfate, well above K_d , it may be assumed that all SBP receptors have captured a sulfate ion.¹² Since the total trapped charge Q_{REC} is equal to the sum of all charges trapped by the receptors, we can write: $Q_{\text{REC}} = \Delta V_{\text{TH}} C_B = ZqN_{\text{REC}}$, where Z is the fractional charge per receptor, q the elementary charge, and N_{REC} is the number of bioreceptors per unit area. N_{REC} was extracted from the AFM data. From the shift in V_{TH} we then estimate the charge per receptor as $Z = -1.7$, which is close to the charge of -2 of a single sulfate ion.

IV. CONCLUSIONS

In summary, we have demonstrated an organic Bio-FET prototype transducer with an integrated SBP. Fluorescence spectroscopy and tapping mode AFM were used to confirm the covalent coupling of the SBP receptor to the surface of a maleimide functionalized polystyrene layer. Measurement of the dissociation constant of SBP after drying and rehydration showed that the protein remains active even after being dried. In the Bio-FET transducer the sulfate ions could be detected by a shift in the threshold voltage. The effective charge per protein is derived as $-1.7q$ per protein.

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